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Article Title: Dual targeting of Hsp90 in childhood acute lymphoblastic leukaemia

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Running Title: Hsp90 inhibition in childhood ALL

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Survival rates for children with acute lymphoblastic leukaemia (ALL) have improved considerably to over 90% in recent years but despite these advances 15-20% of patients relapse. Current chemotherapeutic regimens are designed around the properties of bulk leukaemia cells, which differ from those of the leukaemia initiating cell populations (LIC) (Cox et al, 2009). If drugs have no effect on LIC, these cells may proliferate and cause relapse. Since several populations in childhood ALL have been shown to have LIC properties (Cox et al, 2009; Diamanti et al, 2013) developing therapies that are effective against all leukaemia cells, with minimal toxicity to normal cells, is of utmost importance.

Efforts to uncover the biological pathways that mediate drug resistance and promote cell survival have lead to the targeting of heat shock protein (Hsp)90. Hsp90 is a molecular chaperone protein involved in maturation and stabilisation of a range of oncogenic client proteins, such as Bcr-Abl, Akt and IKK, that are known to be mutated and/or overexpressed in leukaemias (Mjahed et al, 2012). Targeting Hsp90 could have an impact on several oncogenic pathways and use of Hsp90 inhibitors is a promising approach for cancer therapy (Hassane et al, 2008; Hertlein et al, 2010; Lancet et al, 2010; Hong et al, 2013).

Alvespimycin (17-DMAG) targets the binding site of ATP in Hsp90 and has shown clinical activity in acute myeloid leukaemia (AML) (Lancet et al, 2010; Mjahed et al, 2012). Celastrol has been shown to increase tumour necrosis factor-induced apoptosis (Sethi et al, 2007), and disrupt the Hsp90/Cdc37 complex (Zhang et al, 2008). Celastrol significantly impairs viability and engraftment of AML LIC by inhibiting NF-κB survival signals and inducing oxidative stress (Hassane et al, 2008). However, there are no reports on the efficacy of alvespimycin or celastrol in childhood ALL. The aim of this study was to examine the effects of these structurally and functionally distinct Hsp90 inhibitors on primary ALL cells and evaluate their potential when used in combination.
Cells from 3 BCP-ALL, 3 T-ALL and 3 cord blood (CB) cases were incubated with alvespimycin for 24 hours and celastrol for 48 hours then survival was compared (Fig 1A). Clinical characteristics of ALL samples are shown in Table S1. The IC\textsubscript{50} for alvespimycin was reached using 10.2nM in BCP-ALL cases and 43.9nM in T-ALL cases. Celastrol reduced the viability of BCP-ALL and T-ALL cells to a similar extent with IC\textsubscript{50} of 0.8 and 0.8nM, respectively while the IC\textsubscript{50} of CB cells was higher at 2.3nM. For combination experiments celastrol was used at 0.1nM and alvespimycin at 1nM and 10nM (Fig 1B). Both drug combinations significantly reduced the viability of ALL cells, whilst sparing CB cells. Using 0.1nM celastrol with 10nM alvespimycin (Hsp90i) reduced BCP-ALL viability to 30.6±11.2% compared to CB (81.4±8.3%, \( P=0.002 \)), an improvement of 55.8-66.7% over each drug alone. Similar in vitro efficacies of celastrol and alvespimycin have been reported in AML (Hassane et al, 2008) and chronic lymphocytic leukaemia, respectively (Hertlein et al, 2010).

To assess the effects of Hsp90i on LIC and haemopoietic stem cells (HSC), cells from 3 additional BCP-ALL cases were stained with anti-CD34 and anti-CD19 and CB cells were stained with anti-CD34 and anti-CD38 then sorted. Following treatment with Hsp90i, the proportions of surviving unsorted and all ALL LIC subpopulations (CD34\(^+\)/CD19\(^+\), CD34\(^+\)/CD19\(^-\), CD34\(^-\)/CD19\(^+\) and CD34\(^-\)/CD19\(^-\)) were significantly reduced (≤5.8±6.9%) compared to unsorted CB and HSC (\( P≤0.0003 \), Fig 1C). In contrast, unsorted CB and HSC were largely unaffected (90.8±2.0% and 94.7±26.0% surviving). Furthermore, Hsp90i had no detrimental effects on long-term proliferation (Fig S1) or colony formation of CB cells (data not shown).

The effects of Hsp90i on the engrafting capacity of LIC was assessed in NOD/LtSz-scid IL-2Ryc null (NSG) mice (Fig 2A). Treatment with Hsp90i, prior to inoculation, prevented engraftment of unsorted cells and all LIC subpopulations in 2/3 cases. In the third case (pt.
15), engraftment was prevented in NSG inoculated with CD34−/CD7− cells but only reduced in mice with unsorted (68.7%), CD34+/CD7+ (24.1%) and CD34+/CD7− (48.4%) cells. This sample was from a patient in relapse and therefore may be more resistant. Hsp90i treatment did not significantly affect the engrafting capacity of normal HSC (P=0.3, Fig 2B). These data are more promising than what has been reported in AML, where treatment with 2μM celastrol prior to inoculation into NOD/SCID mice reduced but did not prevent engraftment in 2/3 cases (Hassane et al, 2008).

Subsequently, the in vivo efficacy of Hsp90i was assessed in NSG with established disease (≥4% leukaemia in PB, Fig 2C). Interestingly, mice engrafted with T-ALL cells from pt.15 initially responded to 5 doses of celastrol (1mg/kg) alone or in combination with 2 doses of alvespimycin (13mg/kg). However, after 14 days of treatment, leukaemia levels had increased and were similar to the placebo-treated group. Disease burden in NSG engrafted with BCP-ALL cells from pts 7&8 was not reduced by therapy and most animals did not complete treatment. This may be due to high leukaemia burden at commencement of treatment (up to 70% in some cases) and/or insufficient Hsp90i doses. Alvespimycin was used at equivalent doses to that used in patients with advanced AML (Lancet et al, 2010). It may be possible to use higher doses of celastrol, as 5mg/kg has been documented in lung cancer models over a longer time-course than assessed here (Liu et al, 2014). More detailed studies will be required to thoroughly assess the in vivo efficacy of these Hsp90 inhibitors in ALL.

This study represents the first report assessing Hsp90 inhibition both in vitro and in vivo in childhood ALL and the first description of combining two Hsp90 inhibitors to treat cancer. As Hsp90 targeting has a simultaneous impact on signal transduction pathways that are integral for survival and tumour progression, using Hsp90 inhibitors to treat ALL could prove beneficial, particularly as toxicity to normal cells was minimal. While the in vitro data demonstrated significant elimination of unsorted ALL cells and LIC, in vivo studies need to
be refined to determine the true potential of these agents alone and in combination with chemotherapy.
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Authorship

PD processed samples, designed, performed experiments and wrote the report.
CVC processed samples and commented on the report.
JPM facilitated sample collection, collated the clinical data information and commented on the report.
AB conceived and designed the study, performed in vivo experiments and wrote the report.

Conflicts of Interest

The authors have no competing financial interests to declare.
References


Figure 1  Response of normal and ALL cells to alvespimycin and celastrol

(A) Dose response curves of normal CB (n=3), BCP ALL (pts. 1, 2, 6) and T-ALL (pts. 15-17) to alvespimycin (24 hours) and celastrol (48 hours). Data represent mean ± SD. (B) Effects of alvespimycin, celastrol alone and in combination on CB (n=7) and BCP ALL cells (pts. 1-8). Data represent mean ± SD. ** P≤0.01, *** P≤0.001. (C) Cell survival of unsorted cells and LIC subpopulations in BCP ALL cases (pts. 9-11) treated with the 0.1nM celastrol (48 hours) and 10nM alvespimycin (24 hours) in combination. Unsorted CB (n=7) and sorted CD34+/CD38− HSC (n=4) were also tested. Data represent mean ± SD. *** P≤0.001.
Figure 2  Ex vivo and in vivo response of ALL and normal cells to Hsp90i treatment

(A) T-ALL cells from pts. 15, 19 & 20 were sorted based on expression/lack of expression of CD34/CD7 and all subpopulations were treated with the Hsp90i combination 0.1nM celastrol (48 hours) + 10nM alvespimycin (24 hours). Both untreated and treated cells (10^6 sorted and 10^3-10^6 cells from LIC subpopulations) were subsequently inoculated into NSG mice. Graph shows percentage of leukaemia cell engraftment in the recipient BM. (B) CD34+/CD38 CB HSC (n=3) treated with Hsp90i, as above, were inoculated into NSG mice and the engrafting capacity compared with untreated cells. (C) NSG mice engrafted with 10^6 cells from pts. 7, 8 & 15 were treated with either celastrol at 1mg/kg or 3mg/kg i.p. 5 times weekly for up to 3 weeks or alvespimycin 13mg/kg i.v. twice weekly for up to 4 weeks or both drugs in combination. Graphs show levels of leukaemia cells in PB, each line represents an individual mouse.